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Conventional fluorescence microscopy below the diffraction limit with simultaneous capture of two fluorophores in DNA origami

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Abstract

A conventional fluorescence microscope was previously constructed for simultaneous imaging of two colors to gain subdiffraction localization. The system is predicated on color separation of overlapping Airy discs, construction of matrices of Cartesian coordinates to determine locations as well as centers of the point spread functions of fluorophores. Quantum dots that are separated by as little as 10 nm were resolved in the x-y coordinates. Inter-fluorophore distances that vary by 10 nm could also be distinguished. Quantum dots are bright point light source emitters that excite with a single laser and can serve as a label for many biomolecules. Here, alterations in the method are described to test the ability to resolve Atto 488 and Atto 647 dyes attached to DNA origami at ~40 nm spacing intervals. Dual laser excitation is used in tandem with multi-wavelength bandpass filters. Notwithstanding challenges from reduced intensity in Atto labeled DNA origami helical bundles compared to quantum dots, preliminary data show a mean inter-fluorophore distance of 56 nm with a range (14-148 nm). The range closely matches published results with DNA origami with other methods of subdiffraction microscopy. Sub-diffraction simultaneous two-color imaging fluorescence microscopy acronymically christened (SSTIFM) is a simple, readily accessible, technique for measurement of inter-fluorophore distances in compartments less than 40 nm. Preliminary results with so called nanorulers are encouraging for use with other biomolecules.

Keywords

Super-resolution microscopy; diffraction unlimited; fluorescence; localization microscopy; quantum dots; CCD camera; Atto dyes

1. INTRODUCTION

Numerous techniques have been advanced to resolve fluorophores under the diffraction limit of light including. confocal laser scanning microscopy,¹ total internal reflectance fluorescence (TIRF),² scanning near field optical microscopy (SNOM),³ structured illumination microscopy (SIM),⁴ and stimulated emission depletion microscopy (STED).⁵ Localization microscopy is predicated on the concept that the center of intensity of the

photon distribution derived from the point spread function is more accurately measured than attempting to determine photon position from beam width.⁶ Several offshoots of localization have been advanced with resolution of about 20 nm including photoactivated localization microscopy (PALM), fluorescence photoactivation localization microscopy (FPALM), stochastic optical reconstruction microscopy (STORM).^{6–15} Recently simplified versions of localization microscopy with photoswitchable dyes have yielded less expensive arrangements. Yuan et al. used an EMCCD with photoswitchable fluorophores to gain 39 nm resolution.¹⁶ Zhao et al. added imaging buffers to control photoswitchable cycles and a locking camera to control drift for improved resolution to 1 nm.¹⁷ These techniques were designed with the goal of obtaining finely resolved images of entire microscopic fields. However, the goal here is to simplify the method of imaging to measure the distance between 2 proximal fluorophores in alleged separate compartments. The procedure can be approached mathematically with readily available software. For these applications, localization microscopy with a simple conventional fluorescence microscope can resolve quantum dots with 2 separate emissions, 540 and 630 nm, from routine CCD camera images to as little as 10 nanometers.¹⁸ The work has been limited, however, to quantum dots with long fluorescence lifetimes and high quantum yields that could be excited simultaneously with a single laser wavelength. As a foray to image commonly used fluorescent labels, the laser diode array and microscope were modified and DNA origami helical bundles labeled with Atto dyes at each pole are tested.

2. MATERIALS AND METHODS

2.1 Microscope

Modifications made to the previously described microscope system.¹⁸ Two laser drive boards were used to power individual laser diodes at 488 and 635 nm, 150 and 8 mW respectively (Thorlabs, Inc) for duo excitation simultaneously. The laser beams were collimated, centered and focused into a multimode fiber optic patch cable (Thorlabs, Inc.) and the beams reflected with a R01 multiband dichroic nm 25 × 36 mm Bright line (Semrock, Inc.) and focused with a 1.4 numerical aperture 100x objective lens (Carl Zeiss, A.G). Because of the increased image acquisition time required for fluorophores emitting less than quantum dots, extraneous light needed to be minimized. Aligned linear polarizers (Semrock) were added after the fiber-coupler and the emission filter to reduce extraneous, scattered and reflected light. The previously used aluminum sample holder was replaced with a customized black acrylic slide holder, which was finely abraded to eliminate surface reflections and bolted to the stage to mitigate independent vibrations. To eliminate drift a one piece microscope frame was altered to support the stage and camera in a vertical position and accommodate rapid and stable rotation of the microscope. Slides were firmly attached to the stage with resin. The microscope and electronics were mounted on separate air tables to further dampen vibrations. The stage, micrometer, and graduated lockable actuator were previously described.¹⁸ In the system emitted light passes through a 500 nm cutoff long pass filter FEH 0500 (Thorlabs, Inc.) enabling red and green to be visualized simultaneously.

2.2 Image acquisition

Emitted light was captured with a 1.4 MP Nikon DS-Ri1 camera, with multiple images as previously written.¹⁸ Exposure times were adjusted for each image but generally ranged between 100 msec to 4 seconds. The diode laser is controlled and synchronized to the corresponding exposure period. Each pixel corresponds to 55 nm.

2.3 Image analysis

Raw images were recorded as 16 bit RGB tiff files and analyzed in Matlab (MathWorks, Inc.) according to the algorithm that includes separation of bright spots from background, that correspond to the Airy discs of closely paired red and green fluorophores, noise reduction by Fourier transform, and designation of Cartesian coordinates as previously described.¹⁸ The program calculates the inter-fluorophore distances for all green and red quantum pairs in each image. Multiple frames of the same field were analyzed for comparison with the expected inter-fluorophore distances and limited to proximal fluorophores of different colors.

2.4 Nanoruler synthesis

Customized DNA origami helical bundles (Gattaquant, Braunschweig, Germany) were designed with Atto dyes, 488 and 647 nm externally labeled in a linear fashion parallel to the axis of the rods but with each color of fluorophore at opposite poles (Figure 1). The external biotinylated labels were spaced so that the 2 spots were on average 40 nm apart as previously detailed.¹⁹ Prior work has indicated that separation of dyes is 1.25 nm/dye molecules and the average number of incorporated dyes is 10 at each pole giving a spot size of 12.5 nm.¹⁹ Because the synthesis incorporates a random number of dye molecules at each pole the reaction is stochastic with regard to the number and location populated from the 10 rows on the helical bundle by the labels. On a theoretical basis the expected distances from the center of one emitter to another should range between 27.5-65 nm (Figure 1). In practice much greater variation is expected.¹⁹ To promote a flat orientation of the DNA origami, helical bundles were labeled with about 15 molecules of biotin and bound to coverslips with a biotin-neutroavidin-biotin crosslink between BSA and DNA as previously illustrated.¹⁹ The cover glass was placed on a glass slide with an embedding polymer as described (Gattaquant).¹⁹

2.4 Comparison to quantum dots

For comparison of image intensity with six nanometer quantum dots (Sigma-Aldrich, St. Louis MO) the excitation source was the 405 nm, 150 mW, diode laser (Thorlabs, Inc.), 3.8 mm beam diameter, driven by a 250 mA blue laser diode drive board (Thorlabs, Inc.) as previously detailed.¹⁸

3. RESULTS AND DISCUSSION

3.1 Inter-fluorophore distances

In our prior study we used the distribution of inter-fluorophore distances (between red and green quantum dots) and compared fit them to Gaussian curves. The resulting mean and

distribution of localization at each distance is an acceptable method for determining distances that are otherwise unknown. One of our purposes here was to validate the microscope capabilities for distance measurements. Specifically, testing fluorophores separated by known inter-fluorophore distances was a good measure of the accuracy of the system. Nonetheless, because the inter-fluorophore distance were well established it was reasonable to amalgamate the inter-fluorophore localizations and obtain an average. The average interfluorophore distance was 56 nm but was populated over a broad range from 14-148 nm. The majority of measurements were less than 40 nm. The theoretical distance derived from the stochastic labeling in sites oriented vertically on the DNA origami rods should cover a range of about 27.5-65 nm (Figure 1). This assumes that the rods all lie flat on the surface of the BSA cover slip with the interposed biotin-neutravidin-biotin cross link. One would expect that the surface of the coverslip is not completely flat as protein molecules may aggregate to form an irregular surface. In such conditions, the expected localizations would be biased to be less than 40 nm. However, chemically treated BSA has been shown to form a relatively smooth surface with cover glass with only 5 nm variation so the variation would most likely come from the biotin-neutravidin-biotin crosslink and the external label on a biotinylated staple strand that generally joins the fluorophore to the DNA.^{20,21} A greater distance than predicted could result from several situations including aggregation of DNA origami as well as rods bearing one label that are in proximity of another rod (Figure 2). In keeping with these variations the range of inter-fluorophore distances often exceeds that predicted. For example, for 31 nm and 46 nm spaced fluorophores, the published ranges for optimal Gaussian distributions were 25-135 nm and 25-130 nm, respectively.¹⁹ The ranges are close to the data here for DNA origami helical bundles but much broader than obtained previously for quantum dots.¹⁸

3.2 Signal intensity and comparison to quantum dots

In our prior study quantum dots were used as strong point source emitters. Quantum dots are estimated to be 20 times brighter than rhodamine and 2 orders of magnitude more stable.²² One of the goals was to determine if the expected reduced intensity would prevent Atto dyes from being detected in our system. Therefore, the nanorods or helical bundles were designed to maximize the number of fluorophores, an average 10 per spot at some expense in point resolution. The overall spot size produced was about twice that of the quantum dots. The Atto dyes did yield a high percentage of yellow spots for most images (Figure 3) suggesting that the labels were proximal to each other. However, occasional green spots were seen. Isolated colored spots indicate that only one pole of these nanorods were sufficiently labeled. Red spots were rare suggesting the excitation power of the 635 nm laser was not well matched with the blue. Since the 647 Atto dye has been shown to give a more intense signal,¹⁹ and because the 8mW laser was quite effective with quantum dots, this laser was our initial choice. The signal intensity was markedly reduced for the Atto dyes compared to quantum dots (Figures 2 and 3). The reduced intensity also lessened the number of acceptable localizations of individual fluorophore pairs and failed to provide an adequate probability distribution for complete statistical analysis of single fluorophore pairs. However, the mean and range of distances between the Atto attached helical bundles can be compared to quantum dots that have a similar localizing distance (Figure 4). While the nanorulers yielded a greater percentage of overlapping Airy discs, the reduced intensity led

to fewer localizations and a broader range of inter-fluorophore distances. The signal intensity and probability distribution functions may be improved for the nanorulers by a stronger red laser (work in progress).

3.3 Advantages of the subdiffraction simultaneous two-color image fluorescence microscopy (SSTIFM)

In the current rendition, the microscope has been designed for a specific function, to measure the distance between fluorophores that exist in two compartments separated by 40 nm in a single plane. In keeping with the propensity for acronyms for every variation in the field, our technique can be called SSTIFM, particularly apropos for the “stiff arrangement” of the microscope frame, stage, slide holder, and body to prevent drift. SSTIFM is suitable for a number of applications such as distance measurements between 2 molecules each labeled with a unique color to test their relationship within cellular organelles or across membranes. SSTIFM seems particularly amenable to the technique described by Parfitt et al. for very thin plastic sections.²³ Although very high resolution can be obtained using nanogold labeled antibody localization with electron microscopy,²⁴ biologic application of this technique is often restricted. Many commonly used antibodies including many used for diagnostic purposes in diseases such as ocular cicatricial pemphigoid have restricted epitopes. Antibodies don't recognize the epitopes when prepared with fixatives suitable for electron microscopy. Penetration and interference of the embedding resin may hamper reactivity. Immunoglobulins and complement are much more readily detected by immunofluorescence without prior formalin or paraformaldehyde fixation.²⁵ SSTIFM allows rapid evaluation of multiple fluorophores simultaneously. SSTIFM is also appealing for tracking 2 fluorophores at an interface in solution. In this situation photobleaching of fluorophores is vitiated by rapid diffusion of free and protein bound fluorophores that replenish the fluorescent population. The signal intensity issues encountered in the current work would be obviated by numerous diffusable fluorophores. The simplicity of SSTIFM is also attractive. The microscope and routine CCD camera image multiple colors simultaneously without the need for scanning, galvanometers, mirrors, image tracking, image transformation, locking cameras, EMCCDs, or fiducial frames of reference. This greatly reduces equipment costs and the electronic and vibrational noise associated with these processes. Conjoining the stage-camera and microscope frame to an essentially single stiff unit eliminated drift encountered with other microscopes. The CCD sensor detects actual fluorophore colors so that problems dual excitation, and spectral overlap (so called cross talk) are mitigated. The image processing is simple, rapid and automated with actual inter-fluorophore distances reported from the same image and therefore the same reference frame. The data set is readily available for custom quantitative manipulations in MATLAB. SSTIFM can be further improved in our set up. Newer models of the camera used here acquire images more rapidly. Shorter acquisition times should improve the ability to obtain more images within the temporal window prior to bleaching of the fluorophore and improve the data. SSTIFM is computationally and technologically much less intensive than other techniques (e.g. SOFI, SNOM, PALM, SIM, STED or STORM).

3.4. Limitations of SSTIFM

In the current form SSTIFM has definite limitations. While resolution in one plane is about 10 nm with quantum dots,¹⁸ and at least 40-60 nm with Atto dyes, the orthogonal plane with the accompanying z-resolution was not interrogated. This limits the types of specimens that can be examined to dilute fluorophores on a flat surface. A number of remedies are possible. The simplest is to rotate the specimen or microscope with appropriate fiducials. This however would require the ability to determine the precise location of the fluorophores of interest or have a sample where fiducials are not critical, such as a fluid interface. Other approaches might borrow technology from other methods such as a confocal arrangement with pinholes to reject out of focus light or multiphoton excitation to reduce the fluorescence from underlying fluorophores. The hybrid arrangement would undermine the simplicity of SSTIFM. Another current limitation is that no attempt has been made to obtain a detailed image of entire microscopic field. Although computationally demanding, future work will address this challenge.

4. CONCLUSIONS

Sub-diffraction simultaneous two-color imaging fluorescence microscopy is effective for measuring inter-fluorophore distances below 40 nm for robust fluorophores such as quantum dots. Routine labeling with fluorophores of lower quantum yield such as Atto dyes, present challenges in garnering enough images with suitable fluorescence intensity to obtain detailed probability distributions for statistical analysis. However, preliminary data show that Atto dyes attached to DNA origami helical bundles and spaced at about 40 nm can be resolved. Further modifications such as more intense excitation should make SSTIFM applicable for interrogating commonly used fluorophores for molecules localized to organelles or at interfaces below 40 nm. Unlike most methods for so called superresolution, the instrumentation for SSTIFM is within reach of small laboratories.

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Figure 1.

Pictorial rendition of custom DNA origami helical bundles(Gattaquant) shows variation in distances between the externally attached Atto dyes, 488 at the top and 647 at the bottom. Each position may be randomly populated such that each Atto dye molecule may appear in any of 10 possible rows. The average number of Atto labels generally incorporated by this technique at each pole is 10. The overall spot size for each green and red spot on a single helical bundle should be 12.5 nm depending on the number and positions of sites populated. Only 6 of the 12 helical bundles are illustrated for simplicity.

Figure 2.

Fluorescence image of the DNA origami nanorulers embedded in polymer. Most nanorulers yielded yellow spots but some show only a single label (upper right and below shows only green). The yellow spots reflect overlapping red and green Airy discs and the software is programmed for recognizing this threshold and analyzing Cartesian coordinates and inter-fluorophore distances between green and red. The greens spots are subthreshold and are ignored by the software in this analysis.

Figure 3.

Fluorescence image of red and green quantum dots randomly distributed on cover glass for comparison with DNA origami. Note the much greater intensity of fluorescence and dark background compared to Atto dyes. The quantum dots are random distributed so there are relative fewer yellow dots (overlapping Airy discs) for analysis compare to DNA nanorulers. The yellow dots are selectively analyzed by the software and Cartesian coordinates and inter-fluorophore distances reported. For the purposes of this analysis, determining inter-fluorophore distances the software is programmed to ignore the quantum dots that appear as individual green and red spots as select only those with a specified intensity..

Figure 4.

Gaussian distribution of the number of localizations (y-axis) of quantum dots for comparison with the mean inter-fluorophore distance (nm) and the range obtained with 40 nm DNA Origami nanorulers. The mean for quantum dots was 40.1 nm compared to 56 nm for DNA Origami. The range for quantum dots is much narrower than that of DNA Origami (14-148 nm). Figure modified from Figure 5, Glasgow and Ma.¹⁸